TISSUE CULTURE METHODOLOGY

- sterile technique
- media

PLANT TISSUE CULTURE

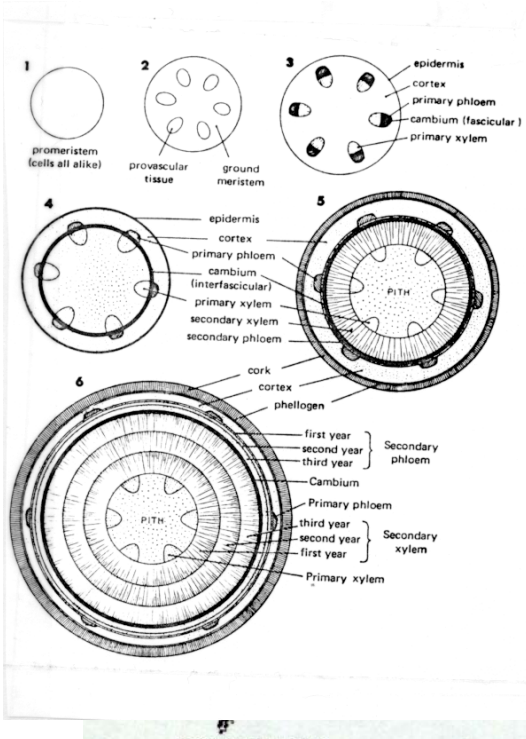
"aseptic maintenance of plant material on a defined medium under defined conditions"

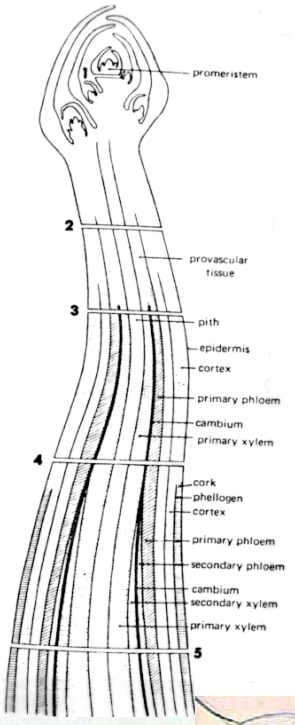
- Plant tissue at all levels of organization (whole plant to single cell) and from a wide variety of sources (for example leaf, stem, root, anthers) will grow in culture.
- The basic steps in tissue culture are:
  1) separate explant from plant body
  2) sterilize the explant
  3) transfer explants to media and maintain cultures in a controlled environment

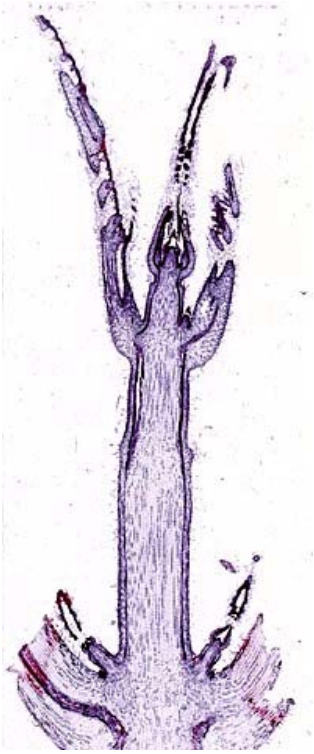
1) Initiation (separation)

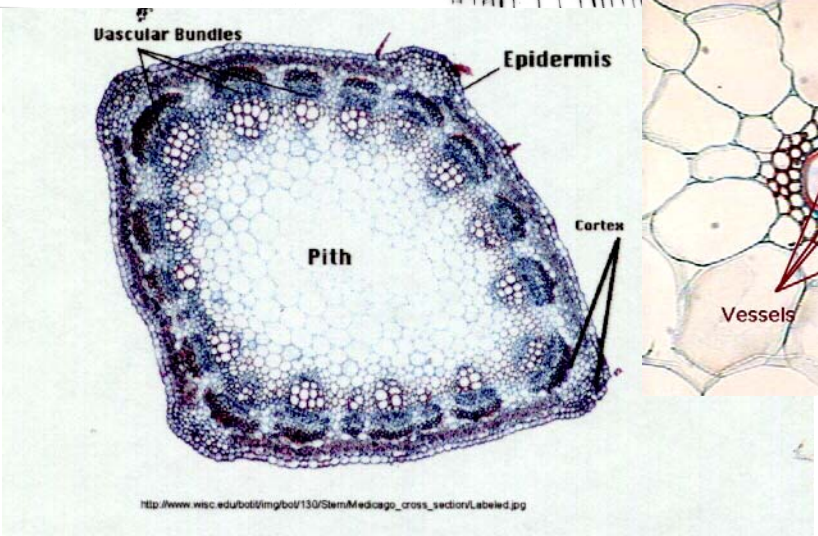
- The piece of plant material placed into culture is called an explant.
- Explanting disrupts interactions among organs, tissues and cells that normally occur in plants including:

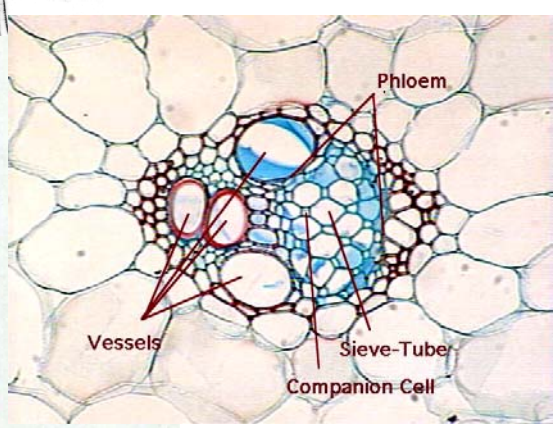
  a) vascular connections providing water and nutrients.



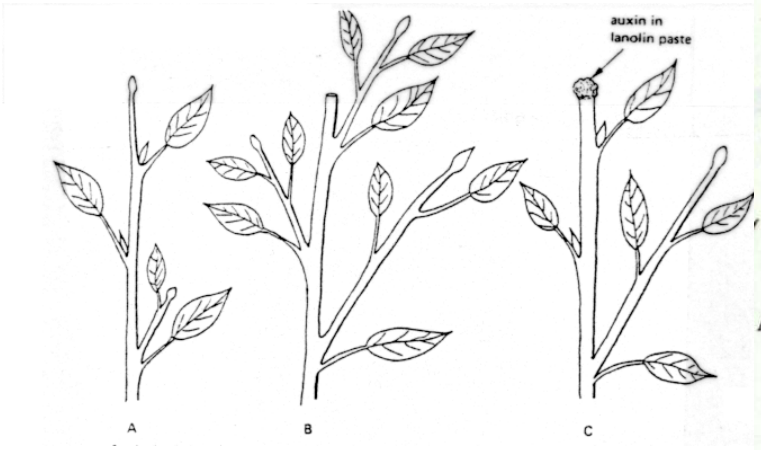


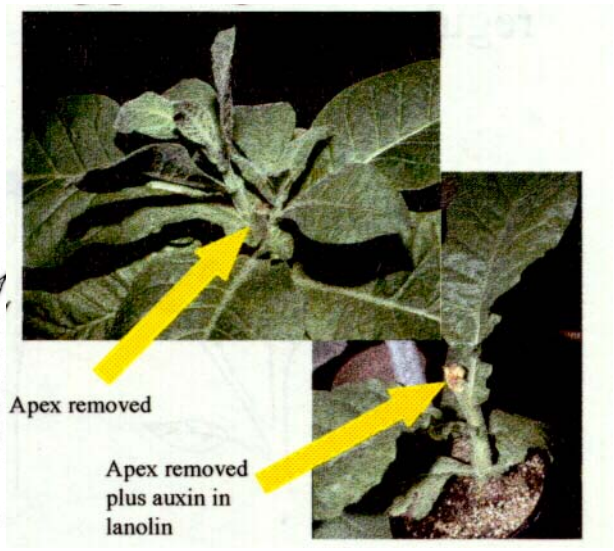






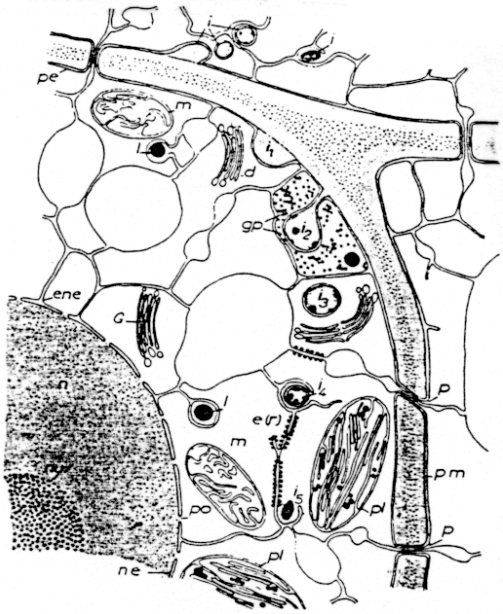
b) vascular connections distributing plant growth regulators:





A. Apical dominance prevents the two upper lateral buds from growing. The lowest one has escaped from the dominance of the apex, and has started growing. **B.** The apex is removed and all the buds have started growing. **C**. The apex is removed and replaced by lanolin paste containing auxin. Lateral bud growth is prevented (except in the lowest bud, which had already broken dormancy and started growing. [After P.M. Ray: *The Living Plant,* Holt, Rinehart and Winston, New York. 1965.]

c) plasmodesmata (cytoplasmic connections between adiacent cells)



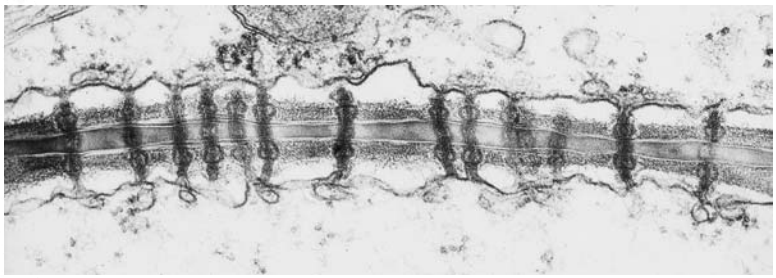


Diagram showing the interpretation of plant ultrastructure and its intercellular relationships. *G*. Golgi complex; *ene*, envagination of nuclear envelope: *er,* endoplasmic reticulum; i_2-i_5, steps in pinocytosis: *l*, lipid, *m*, mitochondrion; *ne*, nuclear envelope; *n*, nucleus; *nu*, nucleolus; *p*, plasmodesmata; *pl*, plastid; *pm*, plasma membrane; *po*, pores.

- All of the connections are interrupted or altered when the explant is made. This allows the tissue to be placed under the control of the tissue culture conditions.

2) Sterilization

- Sterilization of source plant material (or explant), media, culture containers and instruments is required to ensure that contaminants, such as bacteria or fungi, do not establish themselves in the plant cultures.
- Contaminating organisms can affect the way the plant cultures grow or can overgrow and kill the plant cultures

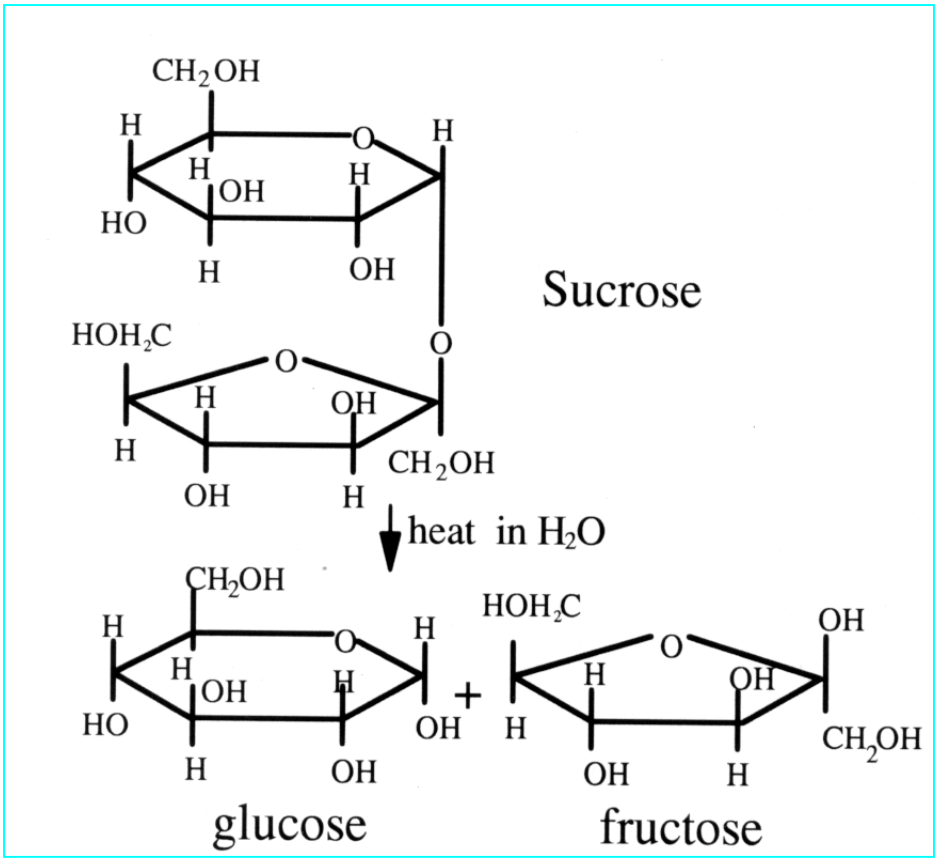
Surface sterilization

- Explants are surface sterilized with a variety of chemicals. The surface sterilizing solutions also commonly contain a detergent (such as Tween), to act as a wetting agent, ensuring that the solution finds its way into nooks and crannies of the plant material.

Surface Sterilizing Agents		
Agent	***Concentration used***	***Time (Min.)***
Calcium hypochloride $Ca(OCl)_2$	9-10%	5-30 min.
Sodium hypochlorite NaOCl	2% of a bleach solution (20% NaOCl)	50-30
Hydrogen Peroxide H_2O_2	10-12%	5-15
Bromide Water	1-2%	2-10
Silver Nitrate $AgNO_3$	1%	5-30
Mercuric Chloride $MgCl_2$	01-1%	2-10
Antibiotics	4-50 mg/l	30-60

Media sterilization

- Tissue culture media are sterilized by: autoclaving with steam at 15 Kg/m^2 (13 kPa) and at 250°F (121°C) for 20 minutes or ultrafiltration through a .22 μm filter.
- Some media components are heat labile and must be sterilized by ultrafiltration; for example:
    1) plant hormones
    2) some vitamins (thiamine)
    3) sucrose (converted to mixture of D-glucose and D-fructose)

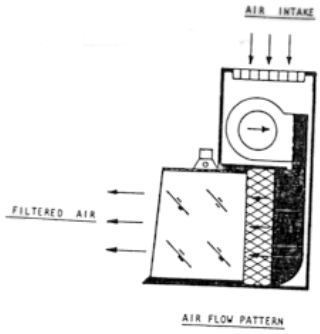


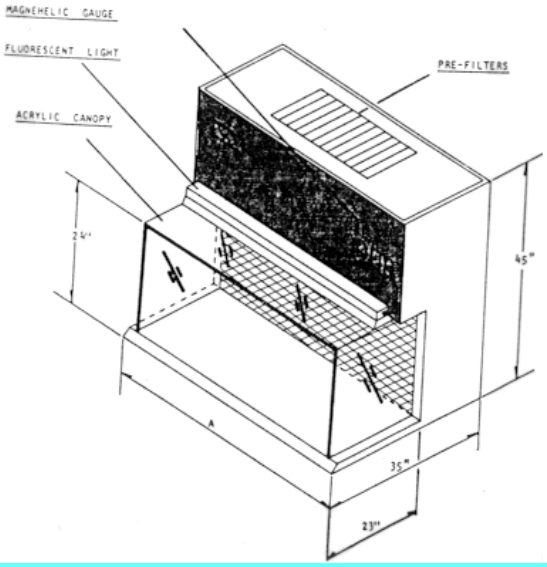
Containers/Instruments

- Instruments and glass culture containers are usually autoclaved with steam (as above) or with dry heat 160°C. (320°F) for 4 h.
- Plasticware is usually sterilized by the manufacturer by exposing it to the gas ethylene oxide.

Sterile Technique

- Sterility is maintained in tissue cultures during manipulations by using sterilized instruments and working a laminar flow bench.
- The surfaces of the flow bench are sterilized by wiping them with alcohol.
- Flow benches filter the air passing over the open cultures through 0.1 μm mesh filters.





3) Maintenance of Cultures

- The emphasis at this stage is to control as many parameters as possible (such as nutrient supply, hormone concentration, light and temperature) - so that results are repeatable.
- The important factors that affect the success of a tissue culture can be divided into:

a) media
- water
- macronutrients
- micronutrients
- carbohydrates
- vitamins
- hormones
- support media
- pH

b) environmental factors
- Temperature
- light

c) explant characteristics
- physiological condition
- genotype

a. Plant Culture Media

- The medium supplies the explant with nutrients and often supports it in the culture vessel.
- Typical plant culture media include: water, inorganic salts, vitamins, carbohydrate, complex organic supplements, growth regulators and in some cases a medium matrix.

Formulations of some plant tissue culture media (mg/l)

	Ingredients	*MS*	*B5*	*White*	*Heller*
Macro-nutrients			134		
	$(NH_4)_2SO_4$				
	$(NH_4)NO_3$	1,650			
	$NaNO_3$				600
	KNO_3	1,900	2,500	80	
	$Ca(NO_3)_2$			300	
	$CaCl_2 \cdot 2H_2O$	440	150		75
	$MgSO_4 4 \cdot 7H_2O$	370	250	720	250
	$NaSO_4$			200	
	KH_2PO_4	170			125
	$NaH_2PO_4 \cdot H_2O$		150	16.5	
	KCl			65	750
micro-nutrients	$FeSO_4 \cdot 7H_2O$	27.8	27.8		
	Na_2EDTA	37.3	37.3		
	$FeCl_3 \cdot 6H_2O$				1.0
	$Fe_2(SO_4)_3$			2.5	
	$MnSO_4 \cdot 4H_2O$	22.3		7	0.01
	$MnSO_4 \cdot H_2O$		10		
	$ZnSO_4 \cdot 7H_2O$	8.6	2	3	1
	H_3BO_3	6.2	3	1.5	1
	KI	0.83	0.75	0.75	0.01
	$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25		
	$CuSO_4 \cdot 5H_2O$	0.025	0.025		0.03
	$CoCl_2 \cdot 6H_2O$	0.025	0.025		0.03
	$NiCl_2 \cdot 6H_2O$				
	$AlCl_3$				0.03
vitamins	*myo*-inositol	100	100		0.03
	nicotinic acid	0.5	1.0	0.5	
	pyridoxine•HCl	0.5	1.0	0.1	
	thiamine•HCl	0.1	10.0	0.1	1.0
	glycine	2.0		3.0	
	Ca D-pantothenic acid			1.0	
carbohydrate	sucrose	30,000	20,000	20,000	20,000
hormones	kinetin	0.01-10	0.1		
	2,4-D		0.1-1.0	6.0	
	IAA	1.0-30			
	pH	5.7-5.8	5.5	5.5	

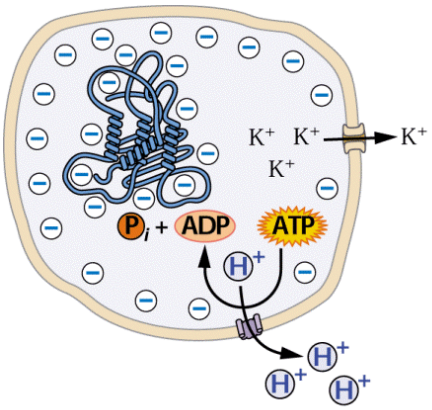
Critical Amounts Required for the Growth of Multicellular Plants (After Stout, 1961)

- The amounts shown are averaged values for many plants and ignore special requirements or requirements exaggerated by competition from other nutrient species.
- Critical amounts denote the amounts that are barely adequate for normal growth and development.
- The upper set are usually referred to as micro-nutrients, the middle set as macronutrients.
- The lower set might not be regarded as nutrients in the usual sense since they are present in the atmosphere or as water, but they are included for sake of completeness.

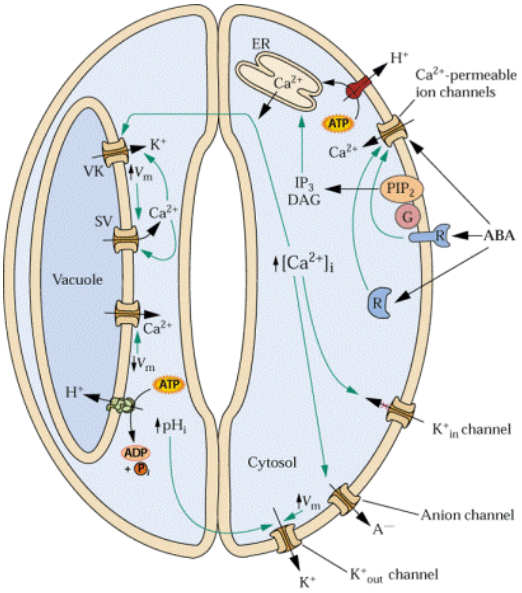
Element	Concentration in Dry Matter		Relative Number of Atoms with Respect to Molybdenum
	atoms/μg	μmoles/g	
Molybdenum	0.001	0.1	1
Copper	0.1	6	100
Zinc	0.3	20	300
Manganese	1.0	50	1,000
Iron	2.0	100	2,000
Boron	2.0	20	2,000
Chlorine	3.0	100	3,000
Sulfur	30	1,000	30,000
Phosphorus	60	2,000	60,000
Magnesium	80	2,000	80,000
Calcium	125	5,000	125,000
Potassium	250	10,000	250,000
Nitrogen	1,000	15,000	1,000,000
Oxygen	30,000	450,000	30,000,000
Carbon	35,000	450,000	35,000,000
Hydrogen	60,000	60,000	60,000,000

i) Macronutrients

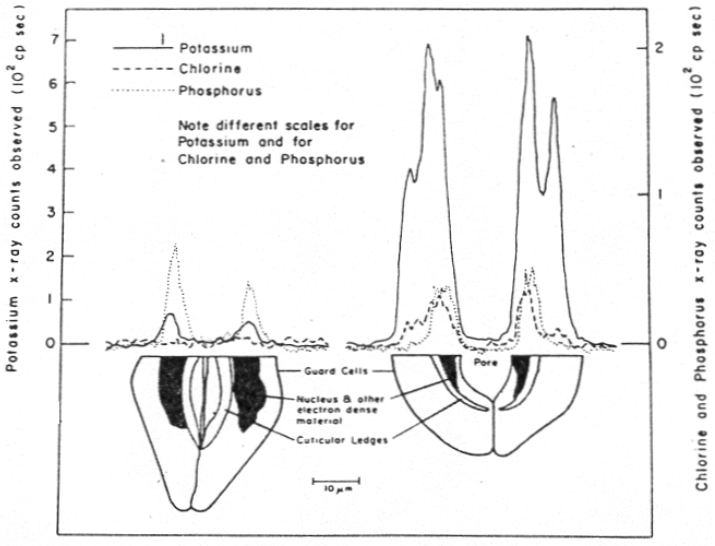
Potassium utilization

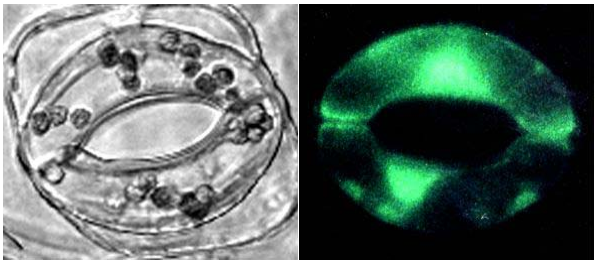


Most proteins have a net negative charge that is countered by K^+ ions. However, because the internal concentration of K^+ is higher than the external solution the tendency is for the ion to leak out of the cell through K^+ channels. This gives cells a net negative membrane potential. The membrane potential can become hyperpolarized by actively pumping H^+ from the cell. Adapted from Biochem Mol Biol of Plants Buchanan, Gruissem, Jones eds, 2000, ASPP



Ca^{2+} signaling coordinates the activities of multiple ion channels and H^+ pumps during stomatal closure. In this model, perception of ABA by the receptor (R) results in an increase in the cytosolic free Ca^{2+} through Ca^{2+} influx or release from internal stores. Increased cytosolic Ca^{2+} promotes opening of plasma membrane anion and K^+ out channels and inhibits opening of K^+ in channels. As more ions leave the cell than enter it, water flows, turgor is lost, and the stomatal pore is closed. From Biochem Mol Biol of Plants Buchanan, Gruissem, Jones eds, 2000, ASPP



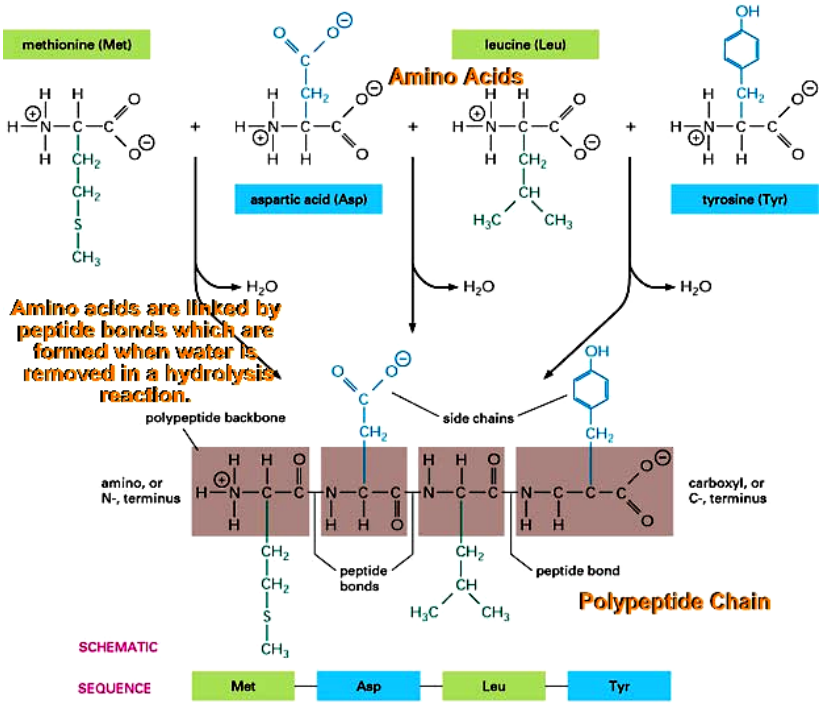


http://www.biology.ucsd.edu/labs/schroeder/protocols/Figure1.jpg

Profiles of relative amounts of potassium, chlorine and phosphorus across an open and a closed stoma. The traces are the result of scanning a 0-5 μm diameter beam across the stomata shown diagrammatically below the traces. In order to indicate the profile scanned, the images of the stomata have been cut off in this diagram where the beam crossed the guard cells. From Humble and Raschke (1971) *Pl. Physiol., Lancaster* 48, 447-53. By permission of the American Society of Plant Physiologists.

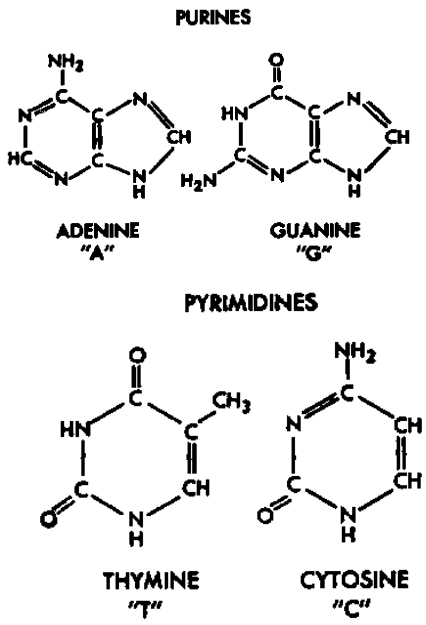
Nitrogen- Containing compounds

Amino acids & proteins



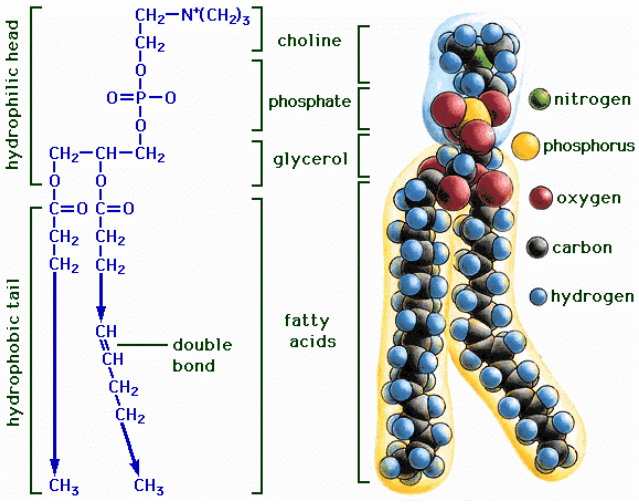
http://crystal.uah.edu/~carter/protein/images/prostruc.jpg

Purine & pyrimidine bases



http://ghs.gresham.k12.or.us/science/ps/sci/ibbio/chem/notes/chpt14/purine.gif

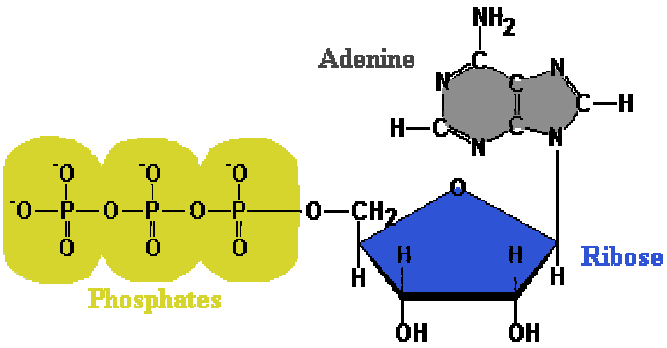
Phospholipid headgroups



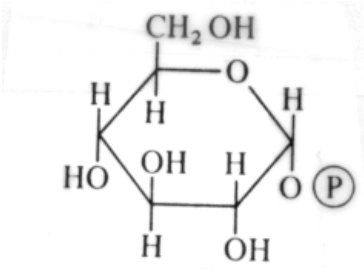
http://www.agen.ufl.edu/~chyn/age2062/lect/lect_06/4_18.GIF

Phosphorus-containing compounds

ATP

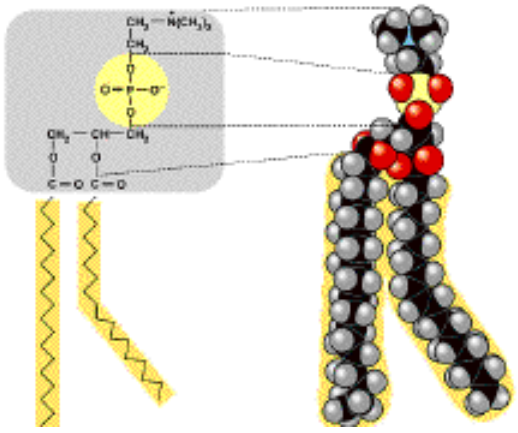


Sugar phosphates



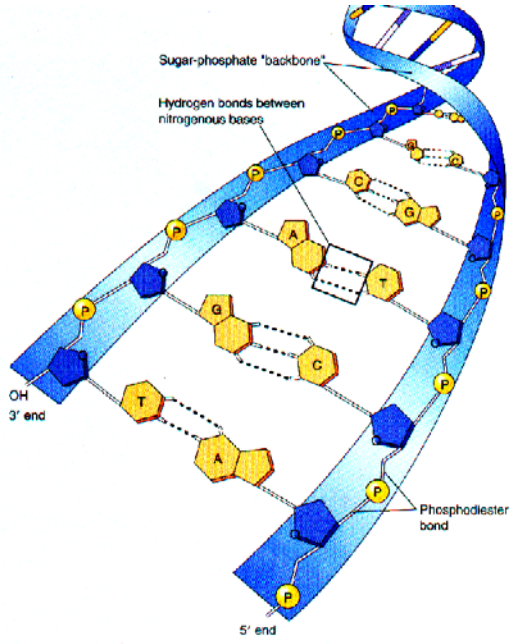
α-D-glucose 1-phosphate

Phospholipid



http://scidiv.bcc.ctc.edu/rkr/Biology201/lectures/Macromolecules/Macromolecules.html

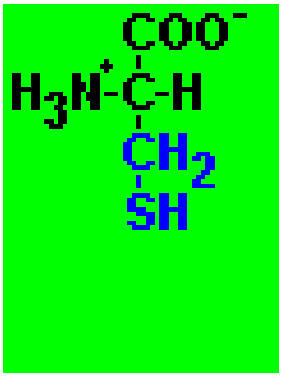
DNA & RNA



http://ghs.gresham.k12.or.us/science/ps/sci/ibbio/chem/notes/chpt14/dna3.gif

Sulfur- containing compounds

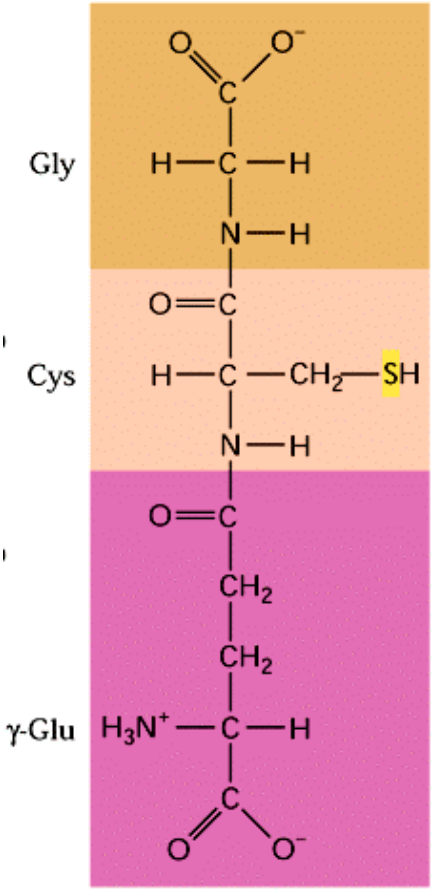
Cysteine



http://www.people.virginia.edu/~rjh9u/aminoacidalpha.html

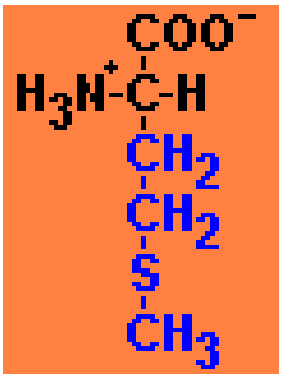
Gluthathione

http://ntri.tamuk.edu/hplc/protein.html



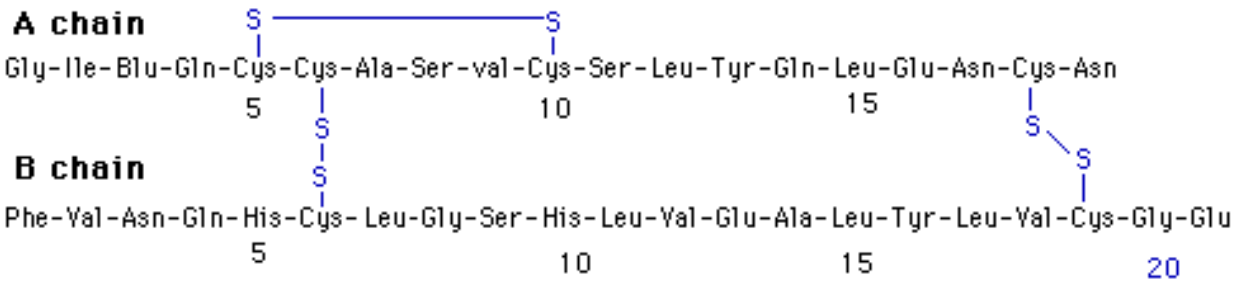
Reduced glutathione

Methionine



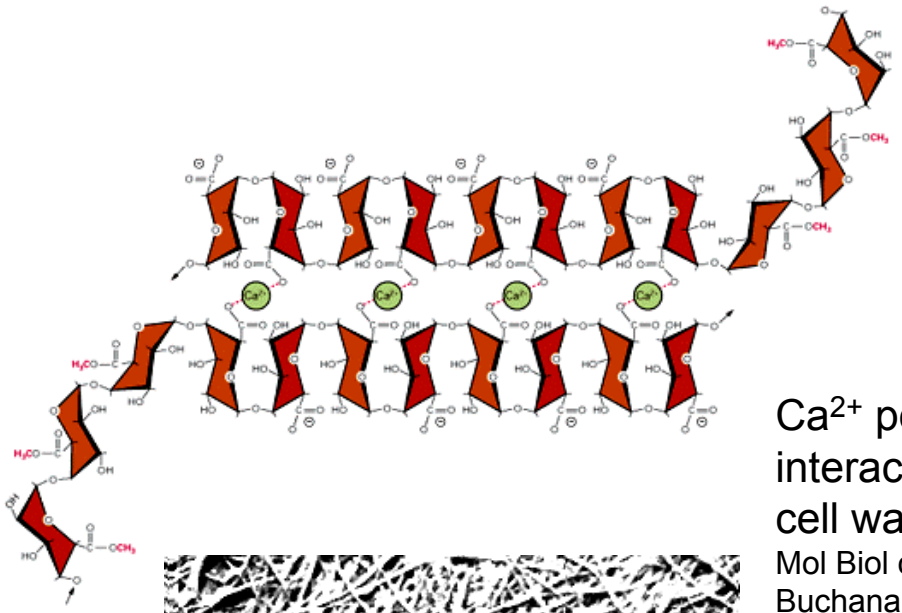
Protein disufide linkages

http://www.agsci.ubc.ca/courses/fnh/410/protein/1_33.htm

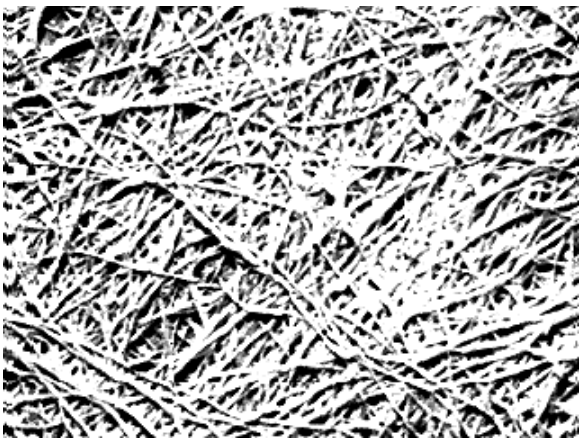


Calcium

- Ca^{++} is required to polymerize pectin in cell walls



Ca^{2+} pectate interactions in the cell wall. From Biochem Mol Biol of Plants Buchanan, Gruissem, Jones eds, 2000, ASPP

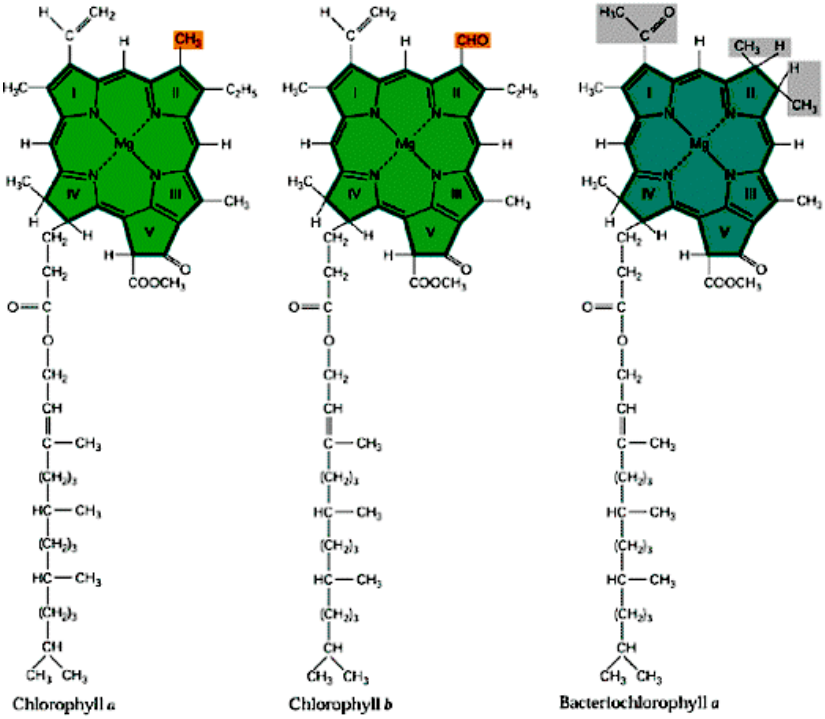


- Ca^{++} is required as a cofactor for enzymatic reactions

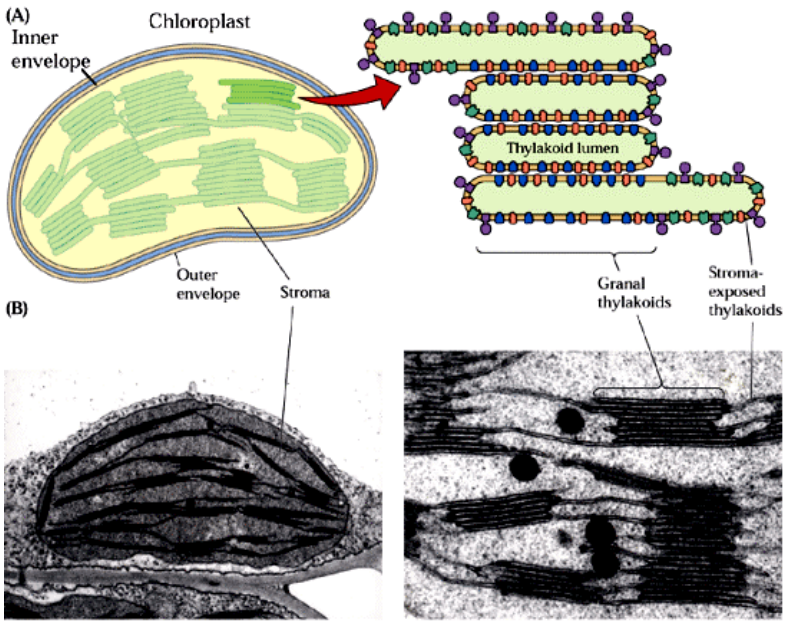
Enzyme Cofactor			
Trivial name	*Source*	*Reaction*	*Metal requirements, etc. (Concentrations M)*
ATPase (Apyrase)	Potato tubers	ATP $\rightarrow$ ADP + Pi	Activated by Ca^{2+}
α-Amylase	Barley	Starch hydrolysis	Ca^{2+} probably needed for activation and stability
Phospholipase D	Cabbage leaves	Lecithin + H2O $\rightarrow$ choline + phosphatidic acid	Ca opt. 0-1; Sr 67%, Ba 34% of activity with Ca^{2+}

Magnesium

- Structural component of chorophyll



From Biochem Mol Biol of Plants Buchanan, Gruissem, Jones eds, 2000, ASPP



- Mg is also required as a cofactor for many enzymes

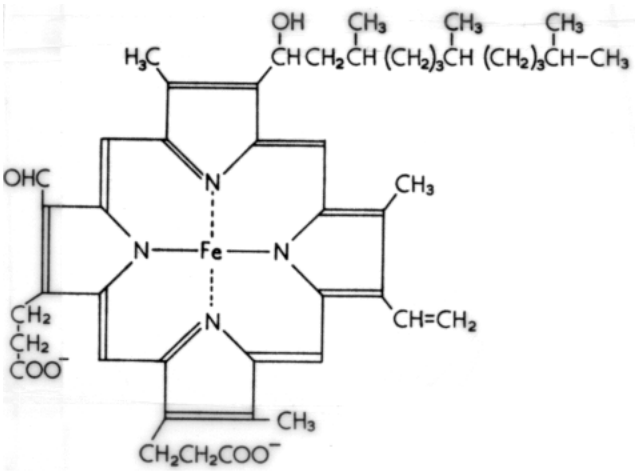
ii) Micronutrients

- Micronutrients are required by plant in μM (or concentrations of less than 100 μg/g dry weight) concentrations.
- There are eight micronutrients included in most plant tissue cultures, including: Fe, Mn, Zn, Mo, Cu, Bo, Ni and Cl.

Major Physiological Functions and Metabolites of Micronutrients Generally Accepted as Essential for All Higher Plants

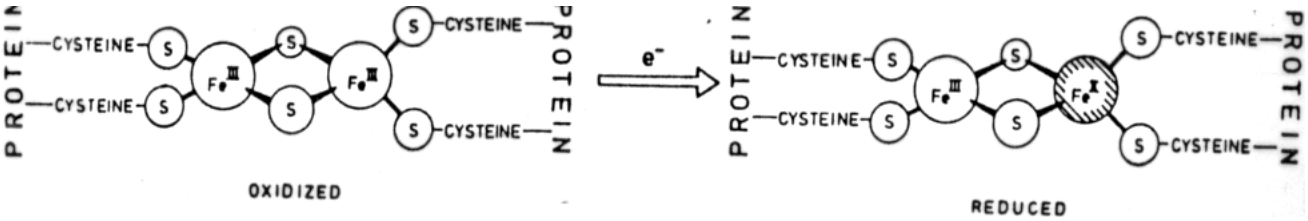
Element	Major functions	Major metabolites
Iron	Biological redox systems (i.e., electron transport chains in photosynthesis and respsiration), enzyme activation, O_2 carrier in N_2 fixation (i.e. leghemoglobin in bacteroids of legume roots)	Heme proteins (e.g., cytochromes, cytochrome oxidase, catalase, peroxidase, leghemoglobin, nitrate reductase), Fe-S proteins (e.g., ferredoxin, aconitase), nonpheme Fe proteins (e.g., lipoxygenase), aminolevulinic acid synthetase, coproportphyrinogen oxidase, various peroxidases (Römheld and Marschner, 1991)
Manganese	Enzyme activation, biological redox systems (e.g., electron transport reactions in photosynthesis), detoxification of oxygen free-radicals, secondary plant metabolite synthesis, structural constituent of ribosomes, disease resistance	Allanloateamido hydrolase, deoxy-b-arabinoheptulosonate-7-phosphate synthetase, kaurene synthetase, phytoene synthetase, water-splitting enzyme S in the Hill reaction, Mn-superoxide dismutase (in mitochondria, peroxisomes and glyoxysomes), NAD malic enzyme and phosphoenolpyruvate carboxylase in C_4 plants (Römheld and Marschner, 1991; Campbell and Nable, 1988; Burnell, 988)
Zinc	Membrane integrity, enzyme activation, gene expression and regulation, carbohydrate metabolism, anaerobic root respiration, protein synthesis, structural integrity of ribosomes, detoxification of superoxide radicals, phytochrome activity (e.g., IAA and gibberellic acid), gene structure (Zn finger motif), disease resistance	Alcohol dehydrogenase, Cu-Zn superoxide dismutase, carbonic anhydrase, aspartate trans carbamylase, tryptophane synthetase, DNA and RNA polymerases, various isomerases, various transphosphorylases (Römheld and Marshner, 1991; Hewitt, 1983; Shkolnik, 1984)
Copper	Physiological redox processes (e.g. photosynthetic electron transport, respiration), detoxification of superoxide radicals, lignification, disease resistance, pollen viability	Cytochrome c oxidase, "alternative" oxidase, Cu-Zn-superoxide dismutase, phenolase, laccase, various amine oxidases, ascorbate oxidase, plantacyanin, plastocyanain, Fraction 1 protein, various blue Cu proteins (Bussler, 1981; Walker and Webb, 1981; Mortvedt et al., 1991)
Nickel	Urea and ureide metabolism, iron absorption, seed viability, nitrogen fixation, reproductive growth	Urease, microbial dehydrogenases, hydrogenases, and methyl reductases (Eskew, Welch, and Norvell, 1984; Eskew, Welch and Norvell,1983; Brown, Welch and Madison, 1990; Brown, Welch and Cary, 1987; Brown et al., 1987; Welch, 1981)
Chlorine	Osmoregulation, charge compensation (i.e.) counter ion in cation uptake), reactivity of enzymes, photosynthesis, disease resistance, stomatal regulation	Conformation of peptides via binding to positive charged sites (e.g., histidyl groups), water-splitting system in photosystem II (Römheld and Marschner, 1991; Hewitt, 1979)
Boron	Cell wall formation and stabilization, lignification, xylem differentiation, membrane integrity, carbohydrate utilization, pentose phosphate metabolism, phenol metabolism and auxin activity, pollen germination and growth, inhibition of callose formation, stomatal regulation	Various cis-diol complexes (e.g., uronic acid and o-diphenols). B-callose complexes (Römheld and Marschner, 1991; Hewitt, 1984)
Molybdenum	Electron transfer reactions, nitrate reduction, nitrogen fixation, ureide metabolism, sulfate oxidation, pollen formation, protein synthesis	Nitrate reductase, xanthine dehydrogenase, sulfite oxidase, nitrogenase (Heweitt, 1984; Römheld and Marschner, 1991)

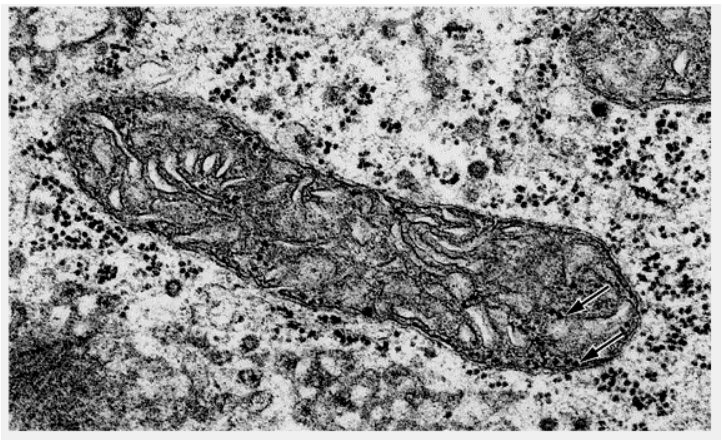
Iron- Containing Compounds - electron carriers

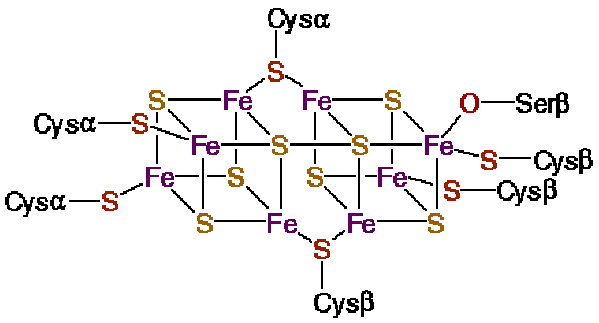


- Cytochromes contain heme groups that have iron atoms that change from Fe_{III} to Fe_{II} when it accepts an electron

- Heme group found in leghemoglobin of nodules
- Iron-sulfur proteins have one or two iron atoms bound to the sulfur atoms of cysteine side chains





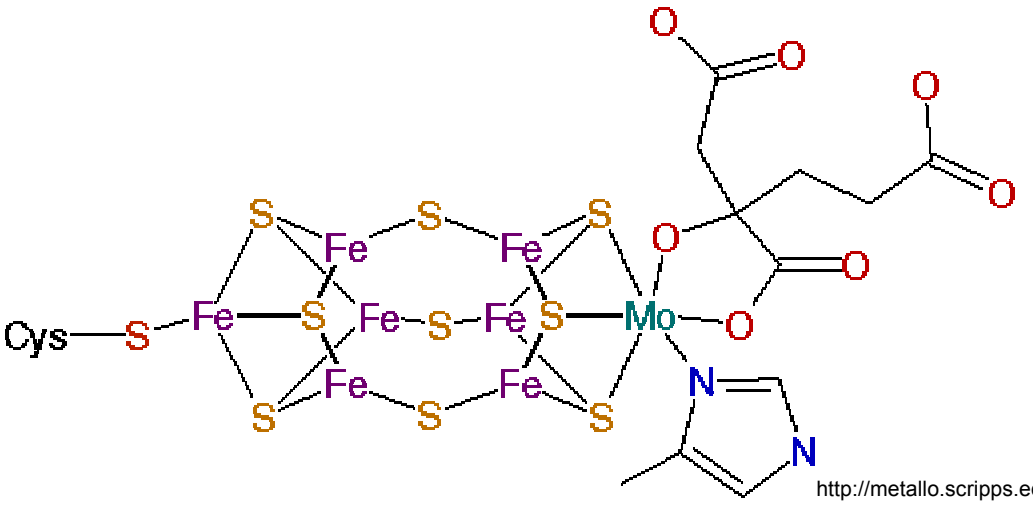


From Biochem Mol Biol of Plants
Buchanan, Gruissem, Jones eds,
2000, ASPP

http://metallo.scripps.edu/PROMISE/Pold_cluster.gif

http://metallo.scripps.edu/PROMISE/NITROGENASE1.html

Mo-Nitrogenase component I (MoFe protein)



http://metallo.scripps.edu/PROMISE/M_cluster.gif

"Biological nitrogen fixation, i.e. reduction of molecular nitrogen to ammonia , is catalysed by the nitrogenase enzyme system (EC 1.18.6.1). Molybdenumnitrogenase (Mo-nitrogenase), which is found in all nitrogen fixing organisms, consists of two components: component I [nitrogenase molybdenum-iron (MoFe) protein, or dinitrogenase], and component II [nitrogenase iron (Fe) protein, or dinitrogenase reductase]

$$N2 + 8H+ + 8e^{-} + 16MgATP \rightarrow 2NH3 + H2 + 16MgADP + 16Pi$$

The MoFe protein is an 2ß2 tetramer; the - and ß-subunits are products of the nifD and nifK genes respectively. The Fe protein is a homodimer, the monomer being coded for by the nifH gene. Nitrogenase binds and hydrolyses 2MgATP, yielding 2MgADP and 2Pi for each electron that is transferred from the Fe protein to the MoFe protein . The MoFe protein contains two types of metal centres, called the M-cluster and the P-cluster. The M-cluster, also termed the iron-molybdenum cofactor (FeMoco), is thought to provide the substrate-binding site." (http://metallo.scripps.edu/PROMISE/NITROGENASE1.html)

iii) Additional Elements

- Other elements are essential for plant growth under specific conditions, for example:
  - Co is required for symbiotic N fixation,
  - Na is required by plants that use the C4 photosynthetic pathway and
  - Si has beneficial effects on a variety of plants.

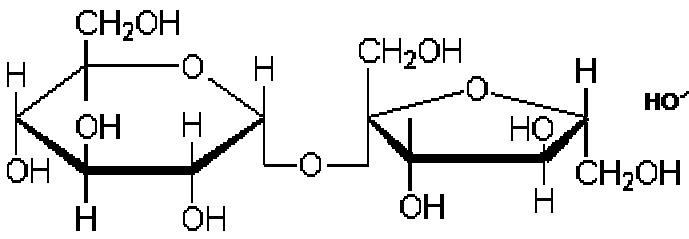
iv) Organic Components of Plant Tissue Cultures

- Three groups of organic constituents found in most tissue cultures including:
  - carbohydrates,
  - vitamins and
  - hormones.
- Some media have additional organic supplements.

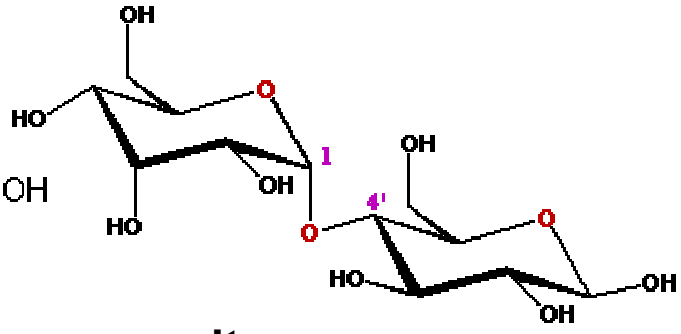
Carbohydrates

- Most plant tissues require some exogenous source of carbohydrates because they do not photosynthesize efficiently.
- The disaccharide sucrose, which is the product of photosynthesis is usually included at 20-40 g/l.
- Sometimes monosaccharides such as: glucose, fructose, galactose, mannose, sorbitol, and ribose are used.
- Arbinose, xylose, mannitol and rhamnose will not support the growth of plant cells in culture.
- The optimum sugar concentration may vary with stage of differentiation in culture.

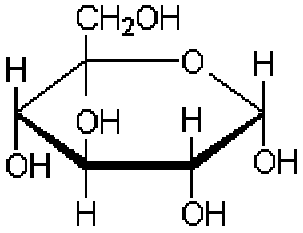
Carbohydrates that support the growth of plant cells in tissue culture.



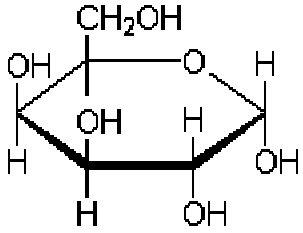
sucrose



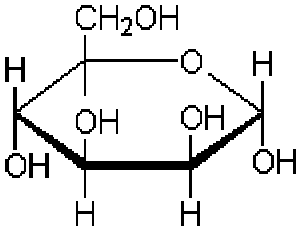
maltose



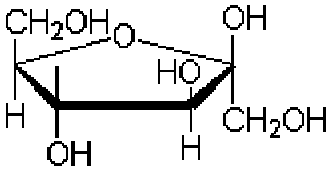
glucose



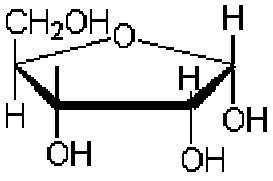
galactose



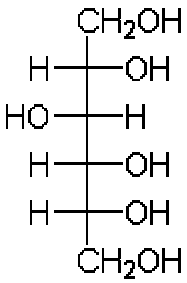
mannose



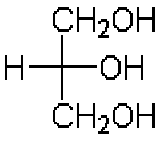
fructose



ribose

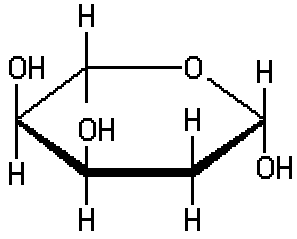


sorbitol

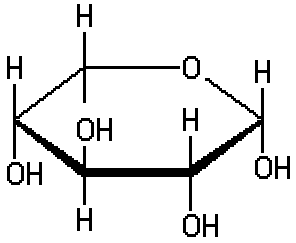


glycerol

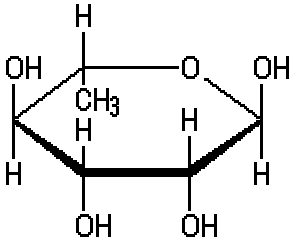
Carbohydrates that won't support the growth of plant cells in tissue culture.



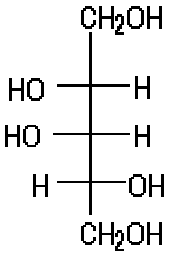
arabinose



xylose



rhamnose



mannitol

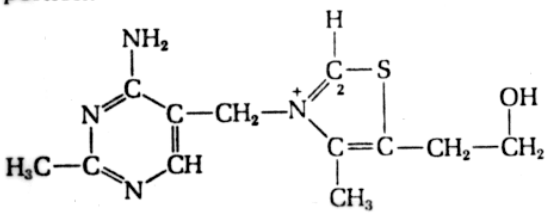
Vitamins

- Function as catalysts in enzyme reactions
- They are only required in small amounts and the ones usually added to cultures include:
  - thiamine (vit B1)
  - nicotinic acid (niacin) and
  - pyridoxine (vit B6).

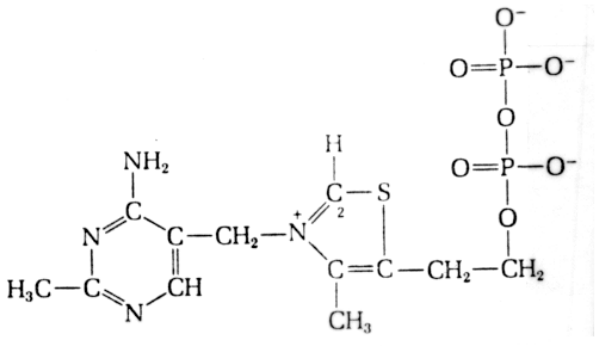
Thiamine

- appears to be universally required

Thiamin and thiamin pyrophosphate. The substituted thiazole is the active portion.

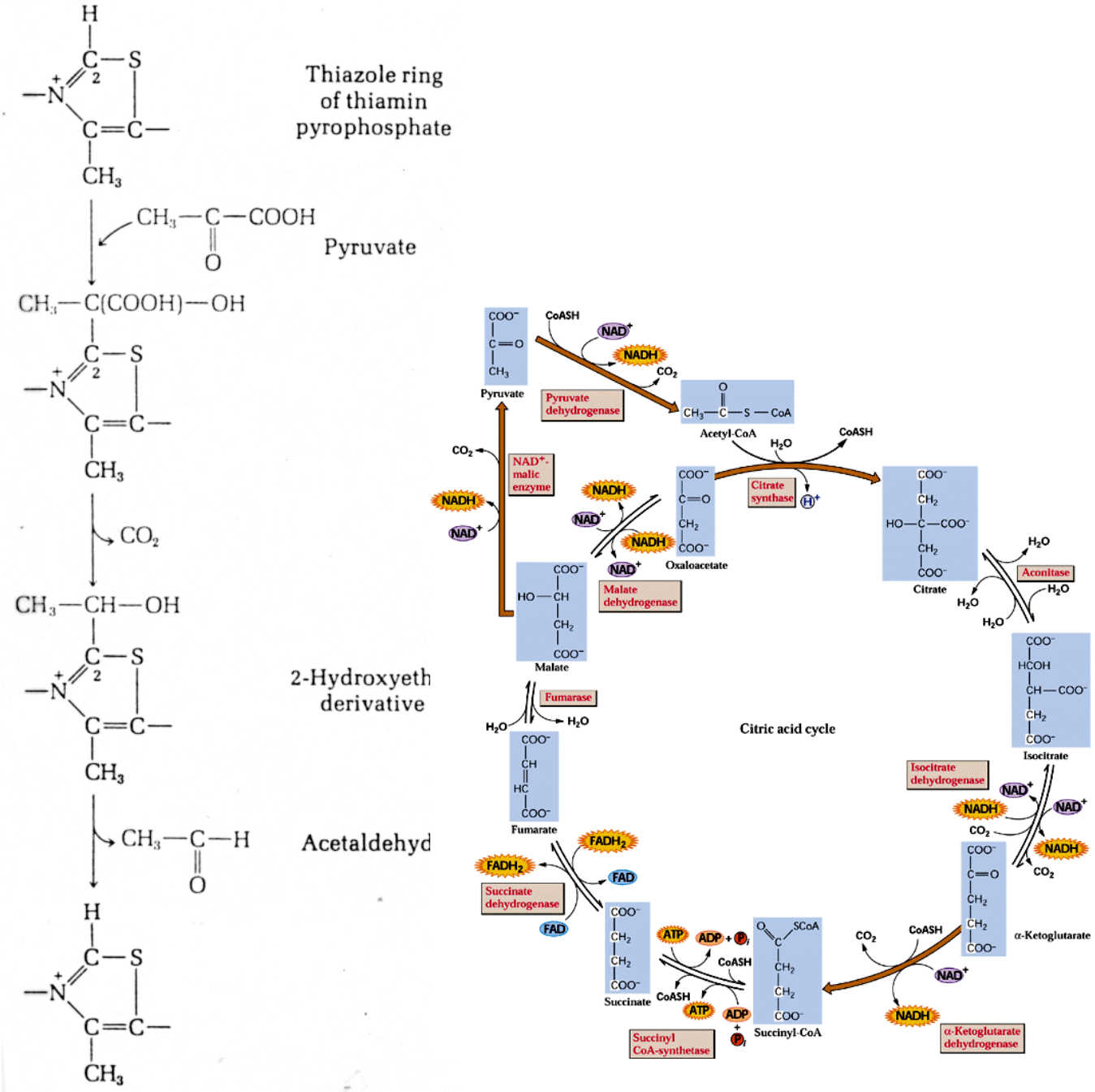


Thiamin (vitamin B_1)



Thiamin pyrophosphate

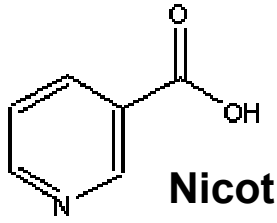
- Thiamine pyrophosphate serves as a coenzyme for reactions in carbohydrate metabolism where aldehyde groups are removed and/or transferred.



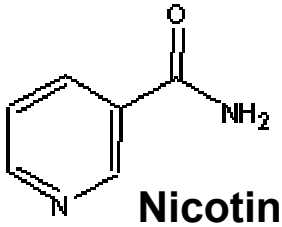
From Biochem Mol Biol of Plants
Buchanan, Gruissem, Jones eds,
2000, ASPP

Nicotinic acid

- Nicotinic acid is synthesized from the amino acid tyrptophan

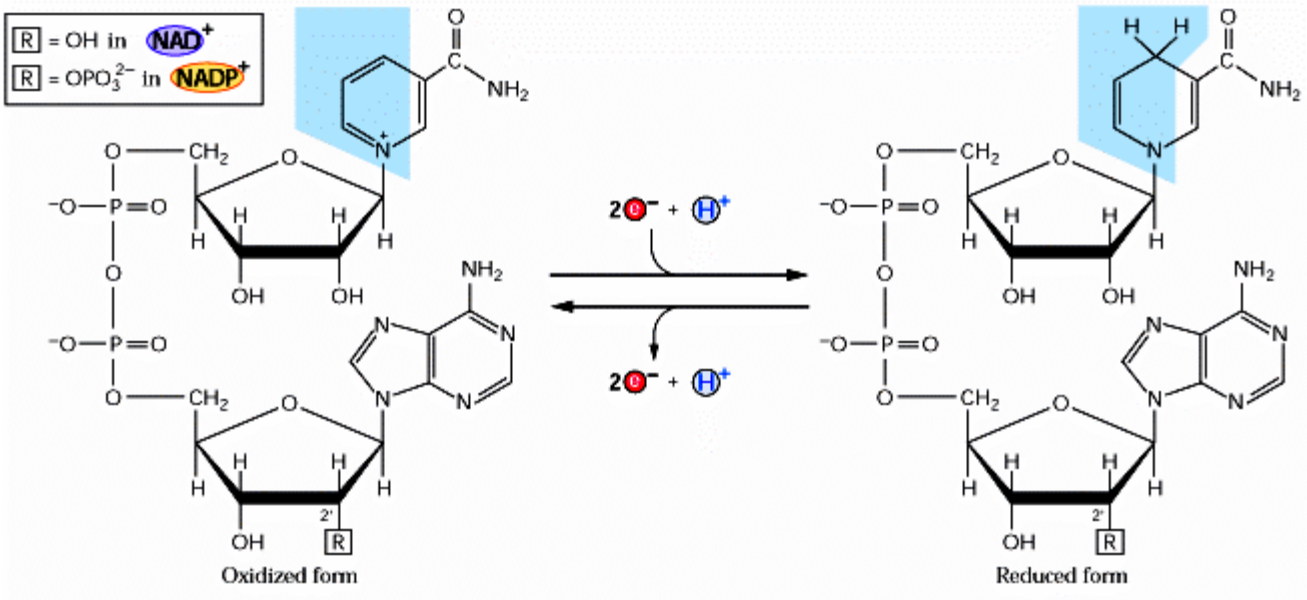


Nicotinic acid



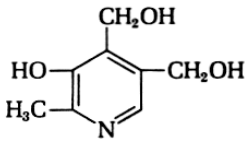
Nicotinamide

- It is an essential component of coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide dinucleotide phosphate (NADP).
- They function as coenzymes for a large number of pyridine-dependent dehydrogenases

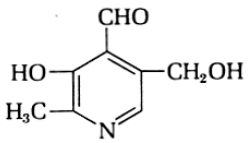


From Biochem Mol Biol of Plants
Buchanan, Gruissem, Jones eds,
2000, ASPP

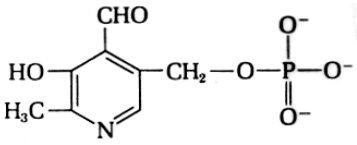
Pyrodoxine (vitamin B_6) and its coenzyme forms



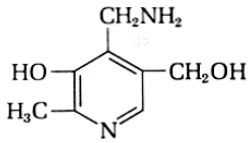
Pyridoxine



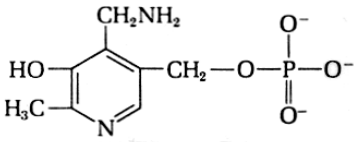
Pyridoxal



Pyridoxal phosphate

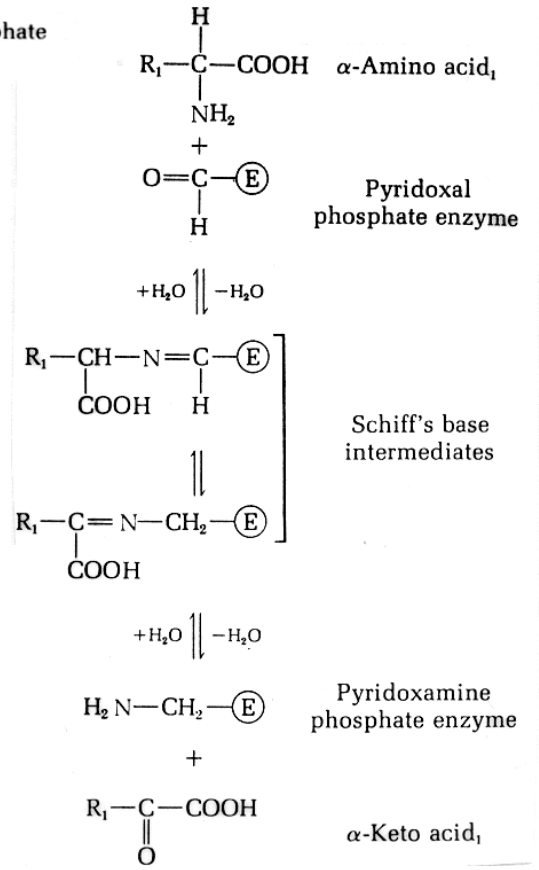


Pyridoxamine



Pyridoxamine phosphate

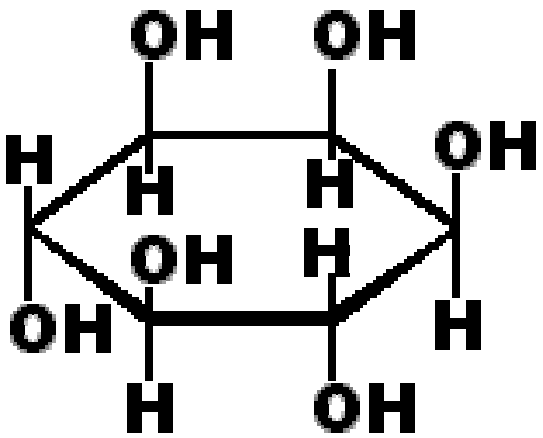
- B_6 is involved in transamidase reactions that are important for amino acid catabolism



Other organic supplements

- A variety of simple and complex organic supplements are used in tissue culture media.
- For example, myo-inositol is seldom essential but its addition can result in dramatic increases in cell growth.
- Myo-inositol is a component of phosphatidyl inositol and phytic acid

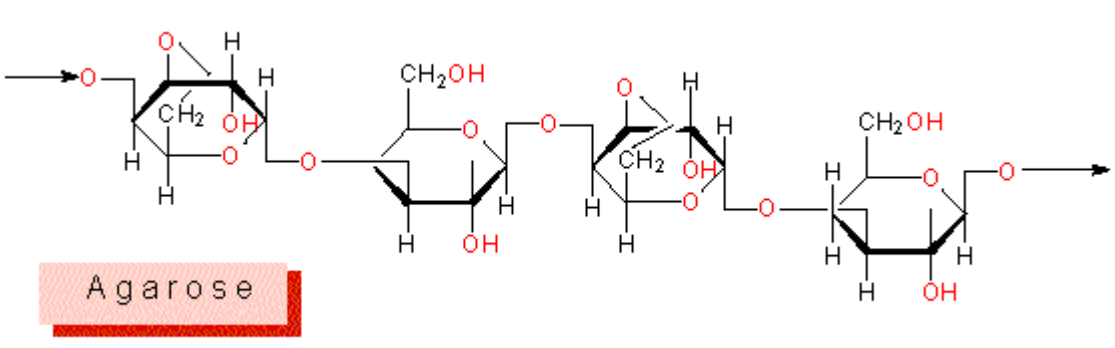
Myo-inositol



Agar

- Agar gum is a gum extracted from red seaweeds (Rhodophyceae, including: *Gelidium cartilagineum* and *Gracilaria con fervoides*) from the coasts of: Japan, Mexico, New Zealand, Portugal, and Denmark.
- Agar is insoluble in cold water but soluble in boiling water.
- A 1.5% solution is clear and when cooled to 45°C it forms a firm, resilient gel that does not melt until 85°C.
- It consists of a mixture of compounds, including a fraction with the greatest gelling ability named agarose and the other fractions named agaropectin.
- Agarose is an alternating copolymer of 3-linked ~-D-galactopyranose and 4-linked 3,6-anhydro-a-L-galactopyranose units.
- Agarose, has a double helical structure that forms a three-dimensional framework that is the proposed method of gel formation.

(http://www.mindspring.com/~earthscents/ECT/gums01.htm)



http://www.rrz.uni-hamburg.de/biologie/b_online/gd26/13.gif

History

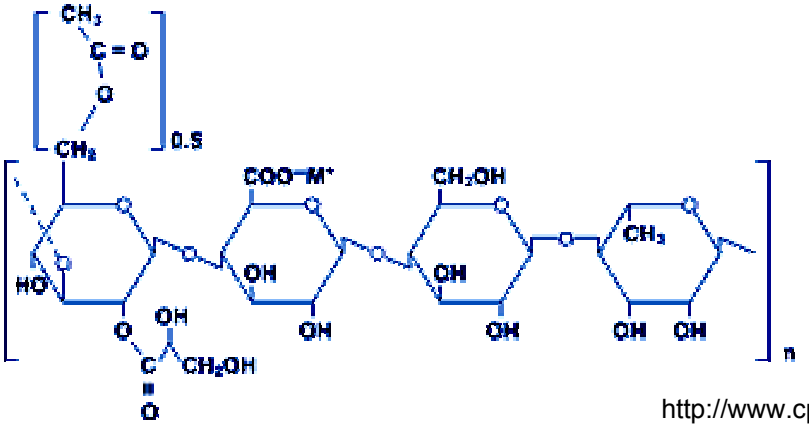
"One day in the year 1658, the Emperor of China was marooned in a snow storm. He took refuge in a nearby hotel. While at the hotel, the innkeeper prepared some seaweed jelly for him. The emperor threw out the remainder of the jelly after he was done with it, into the snow. During the night, the jelly froze. The next morning, the frozen jelly thawed and turned into a dry papery translucent substance. The innkeeper discovered that this substance could be reconstructed back into the jelly. This lead to the discovery of agar (a dried amorphous gelatin-like, non-nitrogenous extract)".
(http://dana.ucc.nau.edu/~lmf2/algae97/reds/agar.html)

- **The freezing method is still used to manufacture agar**

http://www.coastbio.co.nz/images/open_bale_of_weed.jpg

Gelrite®

- Gellan gum, a polysaccharide produced by *Pseudomonas elodea*. Consists of of tetra-saccharide unit, glucose, glucuronic acid, glucose and rhamnose.



http://www.cpkelco.com/gellan/images/structure1.gif

- Forms a transparent gel. The glucuronic acid is neutralized by the presence of potassium, calcium, and magnesium ions. The relative concentrations of these ions controls the gel strength, melting point and setting point.

- The gum has been used as a stabilizer and thickener in foods.

- Has been used as an alternative for agar in tissue cultures

Table 1: Alternatives to agar and gelrite (I)

Alternative:	Applicable to	Specification	Comments/Results	Refs.
Cornstarch (CS)	6 apple varieties, 2 pear varieties and 2 raspberry varieties	0.5g Gelrite + 50.0 g CS, 0.0018 $/g	"more shoot proliferation... than with agar." "..gray-white medium makes it more difficult to detect internal contaminants."	[31], [28]
Tapioca starch (TS)	2 potato cultivars, microshoots	MS-medium and supplements, 8% TS	"TS produced the greatest number of shoots and nodes" "... a good substitue for Bacto agar."	[13]
Barley starch (BS)	Potato tuber discs, differentiation of shoots	MS-medium and supplements, 60.0 g/l BS	"...rapid differentiation of shoots." "...could contain impurities favouring differentiation." "...gets soft during the culture procedure."	[26], [27]
Sago (S), from the stem pith of *Metroxylon sagu*	Nodal segments and shoot tips of Chrysanthenum	MS-based medium, 13% S 0.5 $/kg	"Shoot and root length, leaf and side shoot numbers..on agar-solidified medium significantly higher...", "...as gelling agents...are highly cost-effective compared to agar."	[5]
Isubgol (I), colloidal mucilaginous husk derived from the seeds of *Plantago ovata* (chiefly composed of pentosans), laxative.	Nodal segments and shoot tips of Chrysanthenum, seeds, epicotyl segments, rooting of microshoots of *Syzygium cuminii*, anther culture of *Datura innoxia*	MS-based medium, 3% I 4.0 $/kg	Quantitative result comparision: When agar = 1, I. = 0.86 "The properties of I. ..., good gelling activity even in cold water and reasonable clarity in gelled form, ..are indicative of its potential to become an universal gelling agent in tissue culture media."	[2], [5]

Table 2: Alternatives to agar and gelrite (II)				
Alternative	Applicable to	Specification	Comments/Results	Refs.
Cotton fiber (CF), 100% pure, sterile, nonchlorine bleached rolled fiber	Callus maintenance (a) and shoot organogenesis (b) using *Artemisia* (b), *Agrostis* (a,b) and *Taxus* (a)	CF was compressed, placed in culture vessels (4 cm diameter, 9,5 cm high, babyfood jars). Sterile medium was poured into vessels (25, 16.7 and 10.9 ml/g cotton)	"In terms of callus growth and microshoot proliferation, cotton was a suitable substitute for agar for tissue culture of *Taxus, Agrotis* and *Artemisia*, provided sufficient medium was applied to the cotton."	[18]
Glass wool cloth (GWC)	Nodal segments and shoot tips of Chrysanthenum (*Dendranthema grandiflora*)	GWC cut into stripes (2.5 cm x 15 cm) used as supporting bridges.	"The performance of all the support matrices in terms of length of roots and shoots...were almost identical with those of MS agar medium."	[5]
Nylon cloth (NC)	Nodal segments and shoot tips of Chrysanthenum (*Dendranthema grandiflora*)	NC cut into stripes (2.5 cm x 15 cm) used as supporting bridges.	See GWC	[5]
Polystyrene foam (PF)	Nodal segments and shoot tips of Chrysanthenum (*Dendranthema grandiflora*)	PF cut into blocks (2 cm x 2 cm x 1 cm) used as supporting matrix.	See GWC; "The notable exeption was that P.f.-supported explants produced significantly fewer roots...but longer shoots..."	[5]
Small glass beads (SGB)	Shoot tips of a raspberry, placed as tufts, multiplication; white clover	SGB placed in culture vessels (food container).	"...results in a savings of 60% on media components." "...glass beads allow easy removal of plantlets from the medium.. . The beads can be reused after washing with acid."	[7], [17A]
Filterpaper (FP)	Nodal segments and shoot tips of Chrysanthenum (*Dendranthema grandiflora*)	FP cut into stripes (2.5 cm x 15 cm) used as supporting bridges.	See GWC	[5]

6. References

[1] Adejare, G.O.: personal communication
Virologist, Nigeria

[2] Babbar, S.B. & Jain, N.: "Isubgol" as an alternative gelling agent in plant tissue culture media
Plant Cell Reports 17: 318 - 322, 1998

[3] Baezas-Lopez, P.: Cubans enlist the sun in virus-free propagation
Ceres 156, 15 - 16, 11/12 1995

[4] Bekheet, S.: personal communication
Researcher/Micropropagation

[5] Bhattacharya, P. et al.: Use of low-cost gelling agents and support matrices for industrial scale plant tissue culture
Plant Cell, Tissue and Organ Culture 37: 115 - 23, 1994

[6] Bonaobra, Z.S. et al.: Effect ot table grade sugars on growth and development of Coconut (*Cocos nucifera* L.) embryos in vitro
Poster paper in the 24th International Hortivultural Congress, Kyoto International Conference Hall, Kyoto, Japan, August 21 -27, 1994

[7] Brinks, Th.: personal communication
Biologist/Micropropagation)

[7A] Brinks, Th.: Assembly of a small and cheap laminar air-flow cabinet
Assembly instructions and blueprints, published by Thomas Brinks

[8] Chai-Jang et al.: A study on transplanting grape plantlets in vitro with a lump of soil supporting medium
Acta Agriculturae Boreali Sinica, 10(1): 116 - 119, 1995

[9] Dhamankar, V.S.: Molasses, a source of nutrients for *in vitro* sugar cane culture
Sugar Cane No.4, 14 - 15, 1992

[10] Edwards, M: personal communication
Researcher/Tissue culture & Micropropagation
[11] Ganapathi, T.R. et al.: A low-cost strategy for in vitro propagation of banana
Current Science 68:6, 646 - 65 , 1995
[12] George, J. & Ravishankar, G.A.: Development of modified plant tissue culture media using alternate sources of nitrogen and vitamins for micropropagation
Indian Journal of Experimental Biology 34:2, 163 - 170, 1996
[13] Getrudis, Dj.M. & Wattimena, Ir.G.A.: The effect of agar substitution on micro shoot production of two potato cultivars (*Solanum tuberosum* L.)
Acta Horticulturae 369,9: 447 - 450, 1994
[14] Hossain, M.J. et al.: Tissue culture research at tuber crops research centre
Proceedings of the First National Workshop on Tuber Crops, 28 - 30 May 1989. 1991, 218 - 231.
[15] Jacobsen, H.-J.: personal communication
Professor/Tissue culture & molecular genetics)
[16] Magnaye, L.V. & Escobido, E.O.: Commercial micropropagation of popular local banana cultivars.
Philippine Journal of Crop Science, V.19: p.40, issued May 1996
[17] McCulloch, S.M. et al.: Rooting and establishment of rhododendron microcuttings
Acta Horticulturae 364, 27 - 34, 1994
[18] Moraes-Cerdeira, R.M. et al.: Cotton fiber as a Substitute for Agar Support in Tissue Culture
HortScience 30(5): 1082 - 1083, 1995
[19] Mukherjee, A.: Use of hydroponics in hthe hardening of in vitro plantlets of sweet potato and cassava
J. Root Crops 20(2): 123 - 124, 1994

20] Nyamusa, E. E.: personal communication
Assistant Lab. Technician
[21] Okuno, K. et al.: A trial of improvement of micropropagation of *Brassica campestris* cv. Kukitachina by cost simulation
Journal of the Society of High Technology in Agriculture 8(4), 253 -263, 1996
[22] Peralta, T.B.: personal communication
Senior Sc. Research Specialist
[23] Rajbhandary, S.B.
Agricell report 14:27, 1990
[24] Sharma, T.R. & Singh, B.M.: Simple and cost-effective medium for propagation of ginger (*Zingiber officinale*).
Indian Journal of Agricultural Sciences, 65(7): 506 - 508, 1995
[25] Sipos, J. & Nowak, J. et al.
Am. Potato Journal 65:3, p.53, 1988
[26] Sorvari, S.: Differentiation of potato tuber discs in barley starch gelatinized nutrient media
Annales Agriculturae Fenniale 25: 135 138, 1986
[27] Sorvari, S. & Schieder, O.: Influence of Sucrose and Melibiose on Barley Anther Cultures in Starch Media
Plant Breeding 99, 164 - 171, 1987
[28] Stanley, D.: Tissue-Culturing Plants on Cornstarch
Agricultural Research, p.11, July 1995
[29] Titel, C. et al.: A parenchymatic medium solidifier for plant in vitro culture
Plant Cell Reports 6: 473 - 475, 1987
[30] Voyiatzis, D.G. & McGranahan, G.H.: An Improved Method for Acclimatizing Tissue-cultured Walnut Plantlets Using an Antitranspirant
HortScience 29(1): 42, 1994
[31] Zimmerman, R.H.: Use of starch-gelled medium for tissue culture of some fruit crops
Plant Cell, Tissue and Organ Culture 43: 207 - 213, 1995